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Improved Scoring of Chemical Transformation of C3H/10T1/2 Cells

University of Southern California Cancer Center
Los Angeles

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IMPROVED SCORING OF CHEMICAL TRANSFORMATION OF C3H/10T1/2 CELLS

by

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Grant: R-80-5208

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16. ABSTRACT

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3) The powerful tumor promoter, 12-O-tetradecanoyl phorbol-13-acetate (TPA), produces temporary and reversible rounding up of the cells and loosened adhesion to the substratum.

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5) The cell-surface morphology was studied in the scanning electron microscope (SEM)

6) Preliminary experiments revealed that monoclonal antibodies common to transformed clones, which are probably against oncofetal antigens, can be prepared, and should be useful for scoring transformation.

7) Mouse peritoneal macrophages activated by BCG treatment selectively kill chemically transformed, but not nontransformed C3H/10T1/2 cells.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The development of quantitive and reliable cell culture systems in which chemical carcinogens produce oncogenic transformation of cultured cells has provided powerful new tools for the study of the cellular and molecular mechanisms of chemical carcinogenesis. In addition, these systems provide potentially useful means to screen with reasonable times and cost, the unknown environmental agents for their ability to produce oncogenic transformation. They also provide valuable test-materials for studying the cellular and molecular mechanixms of chemical carcinogenesis.

A number of mammalian test systems have been developed for obtaining quantitive data on the oncogenic transformation of the cultured cells by chemical carcinogens. The C3/1oT1/2 is one of such systems in which permanent lines of fibroblasts derived from mouse prostate are transformed by PAH and quantitated in a focus assay on the top of a confluent monolayer. This document is the

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Director,
Health Effects Research Laboratory

PREFACE

It is now generally accepted that 70-90% of human cancers are environmentally caused. Since our environment contains over 500,000 naturally occurring and manmade chemicals that have not been tested for carcinogenicity, and since it costs about $500,000 per compound for a conventional large scale carcinogenesis test in hundreds of rats and mice kept throughout their lifespan, it would cost $250,000,000,000 to test them all conventionally. This is clearly prohibitive. Thus, the inescapable conclusion is that much quicker and less expensive methods of prescreening must be developed and validated.

The widely heralded Ames test appears to be highly promising as such a prescreen. There is an impressive correlation between the carcinogenic activities of 300 chemicals and their microsome-activated mutagenesis to tester strains of Salmonella typhimurium. However, the correlation is not perfect, there is yet no conclusive proof that carcinogenesis results from a somatic mutation, and (trite as it sounds) bacteria do not get cancer. Hence, it would be folly to rely solely on one prescreening system.

At the present time several systems have been developed for obtaining quantitative data on the oncogenic transformation of cultured cells by chemical carcinogens. It seems clear that such systems, although more complicated and tedious than a bacterial mutagenesis assay, may be more relevant as prescreens for carcinogenic activities. They also provide valuable test materials for studying the cellular and molecular mechanisms of chemical carcinogenesis; such information will be of practical value in providing means to prevent human cancer.

We have developed a system for studying chemical oncogenesis in vitro with the C3H/10T1/2 mouse embryo fibroblasts. However, before this system can be validated as a potential prescreen by testing in it a larger number of carcinogenic and noncarcinogenic chemicals, additional research must be done to perfect its quantitative application.

The perfection and validation of this system that determines and quantitates oncogenic transformation will produce the following benefits:

1) The availability of a reliable pre-screen for environmental carcinogens (or mixtures thereof), which is one of the highest priorities in environmental surveillance.

2) Such a pre-screen can be widely applied to test large numbers of samples (pure compounds and mixtures) of environmental pollutants for their carcinogenic activity.

3) The determination of the oncogenic transformation of cultured animal cells

is much more relevant to human carcinogenesis than measuring mutagenesis in bacteria or other biological or biochemical parameters.

4) The cost, space, and manpower requirements to test individual samples would be expected to be less than 1% of those necessary to carry out adequate tests for carcinogenesis in living rodents.

5) In addition to surveillance of general environmental samples for carcinogenic activity, this system could be used to monitor the changes in carcinogenic activities of specifically collected specimens.

6) The perfection of this pre-screen will also provide important fundamental information on the mechanisms of chemical carcinogenesis that, if intelligently applied, might lead to prevention of many types of human cancer.

7) Such a surveillance could lead to major steps in the abatement of carcinogenic pollutants.

ABSTRACT

This research program was undertaken to improve the scoring of the transformation by chemical carcinogens of C3H/10T1/2 mouse embryo fibroblasts.

1) A probabilistic view of transformed focus formation in these cells induced by methylcholanthrene (MCA) treatment has been formulated and validated. We define P_1 as the probability that a cell will be activated by carcinogen treatment, P_2 as the probability per cell generation that an activated cell will be transformed, and P_3 as the probability per cell generation that an activated cell will be deactivated. The equation derived is

$$\log(F/N) = \log [2p_1p_2 (1-p_3)/2(1-p_3)-1] + n\log (1-p_3),$$

where F = mean no. of foci per dish; N = no. of cells at confluence; and n = no. of cell generations to confluence.

2) 5-Azacytidine induces differentiation of C3H/10T1/2 cells into both muscle cells and adipocytes. Although phorbol-ester related tumor promoters inhibit muscle cell formation, this is not affected by inhibitors of tumor promotion; moreover, other classes of tumor promoters do not inhibit this differentiation. Effects of promoters on adipocyte formation were inconsistent. Hence, this assay cannot be used to screen for tumor promoters.

3) The powerful tumor promoter, 12-0-tetradecanoyl phorbol-13-acetate (TPA) produces temporary and reversible rounding up of the cells and loosened adhesion to the substratum. Although TPA does not affect the growth rate of these cells in 10% fetal calf serum, it does stimulate their growth rate in 1% fetal calf serum.

4) A quantitative study of the "natural history" of clones derived from various morphological types of transformed foci was carried out. All tumorigenic Type III clones formed colonies in soft agarose, this growth efficiency increased with passage number, but only one Type II and no Type I clones grew in soft agarose. Type I clones often progressed to Type II, and many Type II clones progressed to Type III upon further passaging.

5) The cell-surface morphology was studied in the scanning electron microscope (SEM). Nontransformed cells had flat, smooth surfaces. Transformed cells in logarithmic growth exhibited rough surfaces with ruffles, blebs, and microvilli; however, the surfaces of transformed cells post confluence became smooth. "Mini-foci" of rough-surfaced cells were visible within 5 days after MCA treatment.

6) Preliminary experiments revealed that monoclonal antibodies common to

transformed clones, which are probably against oncofetal antigens, can be prepared, and should be useful for scoring transformation.

7) Mouse peritoneal macrophages activated by BCG treatment selectively kill chemically transformed, but not nontransformed C3H/10T1/2 cells.

8) By mutagenesis of a tumorigenic transformed clone of these cells, followed by selection post-confluence at 39.5° with FUdR and extensive cloning, six mutants temperature-sensitive for transformed phenotypes were isolated and characterized.

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PUBLICATIONS RESULTING FROM THIS GRANT

1. S. Mondal and C. Heidelberger. Inhibition of Induced Differentiation of C3H/10T1/2 Clone 8 Mouse Embryo Cells by Tumor Promoters. Cancer Res., 40, 334-338 (1980).

2. C. Boreiko, S. Mondal, K.S. Narayan, and C. Heidelberger. Effect of 12-0-Tetradecanoyl Phorbol-13-acetate on the Morphology and Growth of C3H/10T1/2 Mouse Embryo Cells. Cancer Res. 40, 4709-4716 (1980).

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INTRODUCTION

The development of quantitative and reliable cell culture systems in which chemical carcinogens produce oncogenic transformation of cultured cells has provided powerful new tools for the study of the cellular and molecular mechanisms of chemical carcinogenesis, free of the complex nutritional and immunological milieu of the intact host. In addition, these systems provide potentially useful means to screen with reasonable times and cost, known and unknown environmental carcinogens for their ability to produce oncogenic transformation, as reviewed by Heidelberger (1-3). The first success in this field was achieved by Berwald and Sachs (4), who produced morphological transformation of primary or secondary Syrian hamster embryo cells by polycyclic aromatic hydrocarbons (PAH) in a quantitative clonal assay. This cell strain system has been used extensively in the laboratories of DiPaolo (5), Pienta (6), and Ts'o (7).

A system in which permanent lines of fibroblasts derived from mouse prostate are transformed by PAH and quantitated in a focus assay on top of a confluent monolayer, was first developed in the Principal Investigator's laboratory (8,9), and several important mechanistic studies were done with this cell line (10-14). Subsequently, we established a new cloned mouse embryo cell line, C3H/10T1/2 (15,16), which is now widely studied throughout the world. For example, in the first 5 months of 1980 we furnished C3H/10T1/2 cells to 12 investigators in 4 countries. In this system the nontransformed cells are exquisitely sensitive to postconfluence inhibition of cell division, have an extremely low incidence of spontaneous transformation, and are not oncogenic in immunosuppressed syngeneic mice. Following transformation, they form foci that pile up over a monolayer, grow in soft agarose, and Type III foci produce fibrosarcomas on inoculation into C3H mice (15,16). The cells are transformed by PAH (16) and in a cell cycle-dependent fashion by alkylating agents (17); no endogenous retrovirus (or its core or its RNA) is "switched on" during transformation by a chemical carcinogen (18); transformed clones have individual cell-surface transplantation antigens (19); the C3H/10T1/2 cells activate PAH by mixed-function oxidases (20), metabolize benzo(a)pyrene (BaP) to the 7,8-diol-9,10-epoxide (21), and are transformed by some cancer chemotherapeutic agents (22). We have also succeeded in measuring mutation to ouabain resistance in these cells by various classes of chemical carcinogens (23). C3H/10T1/2 cells have been transformed by X-rays by Terzaghi and Little (24), Miller and Hall (25), and by Han and Elkind (26), who also obtained transformation by neutrons; and by ultraviolet (UV) light by Chan and Little (27).

Other permanent cell lines have been used less frequently for studies of oncogenic transformation. These include BALB/3T3 cells, as developed by Kakunaga (28) and DiPaolo et al. (29); BHK21 hamster kidney cells by Mishra and DiMayorca (30) and Styles (31); and Fisher rat embryo cells infected with a rat leukemia virus, as reviewed by Freeman et al. (32).

Since the discovery of two-stage carcinogenesis on mouse skin (33,34) it now appears that environmental promoters may play as important a role in the causation of some human cancers as do complete carcinogens. Hence, it is important to determine their mode(s) of action and to obtain valid model systems to facilitate their detection. A major advance was provided by Hecker (35), who isolated the active principles of the commonly used croton oil mixture and identified the most potent as 12-0-tetradecanoyl phorbol-13-acetate (TPA). This work has provided a series of related compounds of varying promoting activities for structure-activity studies. Recent studies of mouse skin promotion have been reviewed by Berenblum (36), Boutwell (37,39), and Slaga et al. (38).

The first report on initiation and promotion in cell culture was that of Lasne et al. (40), in which they found that PAH plus TPA accelerated the transformation of rat embryo fibroblasts. We discovered in C3H/10T1/2 cells that the phenomena of initiation and promotion could be reproduced in formal analogy with the mouse skin situation (41); PAH (41), UV light (42), and X-rays (43) act as initiators for phorbol ester promotion, and saccharin is a weak promoter after PAH initiation (44). This formal demonstration of the processes of initiation and promotion in C3H/10T1/2 cells makes our system a valid model both for the screening of environmental tumor promoters and for the study of their mechanisms of action. We have reviewed our work on two-stage transformation (45-47).

In the C3H/10T1/2 cell system it is, unfortunately, impossible to score for oncogenic transformation in early colonies, as can be done with the hamster embryo fibroblast system (4-7). Instead, in our case it is necessary to wait six weeks until transformed foci have piled up on top of a confluent monolayer (16). A number of other properties have been proposed as additional markers of transformation, and during the first three years of this grant we have investigated several of these and found that they could not be applied to improving and shortening the time required for the bioassay. One property, the loss of anchorage dependence of transformed cells as determined by their growth in soft agarose, correlates quite well with tumorigenicity in this system (48, and Progress Report), but cannot be used for initial scoring. It is now clear in the hamster embryo cell system that transformation occurs in discrete stages, in analogy with the well-known phenomenon of in vivo tumor progression as described by Foulds (49). Whereas in the hamster embryo cell system morphological transformation is seen first, followed in time sequentially by the acquisition of fibrinolytic activity, ability to grow in soft agar, and finally tumorigenicity (7), there are indications from our laboratory (see Progress Report) and from Saxholm (50) that in addition to the stages of initiation and promotion (41-44), there are additional stages required for C3H/10T1/2 cells to grow in soft agarose and become tumorigenic. Hence, in this system the acquisition of various criteria of oncogenic transformation may occur at different times in the overall process.

Vasiliev et al. (51,52) were the first to observe with the SEM that in transformed cells there is an alteration in the structure of the lamellar cytoplasm. Porter et al. (53) first demonstrated that there are dramatic changes in the cell surfaces of virally transformed BALB/3T3 cells as compared to their nontransformed counterparts. The nontransformed cells were flat with a smooth surface, whereas the transformed cells had various sizes and shapes with numerous microvilli, spherical blebs, and ruffles on their surfaces. They found that some of these surface changes also occur when

nontransformed cells undergo mitosis (54). The above changes were observed both in viral and spontaneously transformed cells and were attributed to changes in the structure and arrangement of the cytoskeleton in transformed cells. Most of such observations arose from qualitative assessments based on major changes in surface morphology. Few studies, if any, have attempted quantitation, especially in regard to assessing the number of cells with such surface morphology changes with a view to establishing an index for oncogenic transformation. In this regard, it is important to establish what alterations are significant (qualitatively and quantitatively) in individual cells. This we will attempt to do. Our task is complicated by the knowledge that normal cellular events such as cell cycle changes and culture conditions (treatment with trypsin, alterations in pH, levels of dibutryl cAMP, cell density, use of fresh or conditioned culture medium, etc.) affect cell morphology. Quantitative assessment under identical preparative conditions of transformed vs. nontransformed cells should remove the complexities of such influences and permit us to obtain meaningful correlates for oncogenic transformation. Similar changes in the surface morphology of chemically transformed C3H/10T1/2 cells were observed in the SEM by Malick and Langenbach (55) and by Saxholm and Reith (56). The use of antibody coated microspheres as tools for the SEM localization of cell-surface antigens has been recently proposed by Rembaum and Dreyer (57), and we propose to use this new technology.

It is now generally accepted that chemically induced sarcomas in mice and some other types of chemically induced tumors contain cell-surface tumor-specific transplantation antigens (TSTA) that are individual and not cross-reacting (58-60). Although these were originally detected by tedious tumor transplantation experiments, methods have been developed to detect them in vitro by lymphocyte-mediated cytotoxicity (61) and indirect immunofluorescence (62). Such TSTA's have been detected in our laboratory on transformed, but not on nontransformed mouse prostate fibroblasts (11,12) and on C3H/10T1/2 cells (19); they are also individual and not cross-reacting. Hence, these antigens would not be suitable as generally applicable markers for transformation.

In contrast, fetal antigens have now been detected on the surfaces of a number of chemically induced and spontaneous tumors, and these oncofetal antigens are in general common and not individual (63-68). We have also demonstrated the presence of common oncofetal antigens in several clones of chemically transformed C3H/10T1/2 cells (19). The fact that the nontransformed cells do not exhibit such oncofetal antigens may seem surprising in view of the fact that these cells were derived from mouse embryos, but it was shown that some of these cell-surface embryonic antigens occur for only a short time during embryonic development (60). Apparently the antigen was not expressed at the time the original mouse embryo culture was set up. Very recently a new widely cross-reacting antigen (Gp53) has been described as being present on the surfaces of cells, regardless of whether they were transformed by chemical carcinogens, by irradiation, or by some viruses (70). It appears that the oncofetal antigen that is common to chemically transformed C3H/10T1/2 cells is potentially an excellent marker for transformation that could be accurately detected and quantitated.

The development by Kohler and Milstein (71) of the techniques for the production of monoclonal antibodies from hybridomas has revolutionized the field of immunology, and such monoclonal antibodies are already being used as exquisitely specific and sensitive reagents in a variety of fields. This method should be directly applicable to

quantitating the transformation of C3H/10T1/2 cells and providing unique information on the kinetics of the transformation process.

In 1972, Hibbs et al. (72) discovered that peritoneal macrophages harvested from mice treated with BCG were "activated" to kill tumor cells by a relatively nonspecific mechanism that does not involve the conventional immune responses. By using a series of nontransformed and spontaneously transformed mouse embryo fibroblasts as the targets and in vivo activated peritoneal macrophages in an assay involving cytological examination of fixed and stained preparations, the same authors postulated that activated macrophages are primary antineoplastic surveillance cells (73), and they have expanded upon this concept in a review (74). The assay of cell lysis has been quantitated by prelabeling the target cells with ^{51}Cr (75), ^{3}H-thymidine (76), or ^{125}I-5-iodo-2'-deoxyuridine (^{125}I-IUdR) (77) and measuring the release of radioactivity. The latter label has the advantage of sensitivity and the fact that, unlike thymidine, IUdR is not re-utilized for DNA synthesis. It is also possible to activate the macrophages in culture (78).

CONCLUSIONS

All work supported by this grant was aimed at improving the scoring, and making it more rapid and quantitative, of the C3H/10T1/2 mouse embryo fibroblast system for oncogenic transformation and its initiation and promotion. The following conclusions have been drawn:

1) A probabilistic view of transformed focus formation in these cells induced by methylcholanthrene (MCA) treatment has been formulated and validated. We define p_1 as the probability that a cell will be activated by carcinogen treatment, p_2 as the probability per cell generation that an activated cell will be transformed, and p_3 as the probability per cell generation that an activated cell will be deactivated. The equation derived is

$$\log(F/N) = \log [2p_1p_2 (1-p_3)/2(1-p_3)-1] + n\log (1-p_3),$$

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5) The cell-surface morphology was studied in the scanning electron microscope (SEM). Nontransformed cells had flat, smooth surfaces. Transformed cells in logarithmic growth exhibited rough surfaces with ruffles, blebs, and microvilli; however, the surfaces of transformed cells post confluence became smooth. "Mini-foci" of rough-surfaced cells were visible within 5 days after MCA treatment.

6) Preliminary experiments revealed that monoclonal antibodies common to transformed clones, which are probably against oncofetal antigens, can be prepared, and

should be useful for scoring transformation.

7) Mouse peritoneal macrophages activated by BCG treatment selectively kill chemically transformed, but not nontransformed C3H/10T1/2 cells.

8) By mutagenesis of a tumorigenic transformed clone of these cells, followed by selection post-confluence at 39.5° with FUdR and extensive cloning, six mutants temperature-sensitive for transformed phenotypes were isolated and characterized.

Several of the initial objectives have been realized, and sufficiently promising preliminary data have been collected to make it highly likely that continuation of this research will lead to the accomplishment of all the objectives.

RECOMMENDATIONS

(The numbers of these recommendations pertain to the numbers of the conclusions previously stated).

1) The probabilistic view of transformed focus formation of C3H/10T1/2 cells following chemical carcinogen treatment has been derived and validated. This is expected to have major impact on the quantitation and interpretation of transformation in this transformation system that is now widely used throughout the world. We will apply for separate funding to continue this research.

2) The effects of tumor promoters on the 5-azacytidine-induced differentiation of C3H/10T1/2 cells into muscle cells and adipocytes may be useful in establishing the mechanism of action of phorbol ester-related tumor promoters. However, inconsistent results preclude this from being used as a short-term rest for tumor promoters. Hence, this approach has been discontinued.

3) Since the transient rounding-up of cells following TPA treatment does not occur on repeated treatments, which are required for promotion of transformation, this effect is unlikely to be involved in tumor promotion. The growth-stimulating effect of TPA in 1% fetal calf serum seems unlikely to be involved in the mechanism of promotion because promotion experiments are carried out in 10% fetal calf serum in which no growth stimulation was observed. Hence, these approaches are being discontinued.

4) The study of the "natural history" of transformed foci has been complicated, and further studies are required to fully assess the significance of the various transformed phenotypes.

5) The appearance of "mini-foci", detectable in the scanning electron microscope (SEM) only a few days after chemical carcinogen treatment, is a highly promising lead to improve the rapidity of scoring. This led to an application to the EPA for continuation of this research, which is currently funded as EPA grant No. R-808309, and the research continues.

6) The preliminary finding that monoclonal antibodies can be prepared that react with various transformed clones, is extremely promising. The antibodies can be radioiodinated and rapid, objective, and highly sensitive radioimmunoassay can be developed for transformation. Alternatively, the antibody can be labeled with fluorescene and transformed cells can be visualized in a fluorescence microscope and can be separated in a fluorescence activated cell sorter. This is also currently funded on EPA grant R-808309, and the research continues.

7) Although activated mouse peritoneal macrophages selectively kill chemi-

cally transformed cells, it is highly unlikely that this test can be made sufficiently sensitive to be used as a primary means of scoring for transformation.

The successes of the approaches described in 1, 5, and 6, together with interesting data collected from the research described in 2, 3, 4, and 7 indicate that the results obtained on this grant, R-86-5208, fully justify its funding by the U.S. Environmental Protection Agency.

MATERIALS AND METHODS
EXPERIMENTAL PROCEDURES

All cell culture media and heat-inactivated fetal calf serum were obtained from GIBCO. The development of the C3H/10T1/2 cell line, its passaging, and determination of cytotoxicity and transformation were done as described (15,16). The initiation and promotion experiments were carried out as described (41). Additional methods and details of the experiments are provided in the text, legends to tables and figures, and in the accompanying manuscripts.

RESULTS AND DISCUSSION

a. Probabilistic Theory of Transformed Focus Formation

One of the original aims of the project was to apply to C3H/10T1/2 cells the transformation of single individual cells in dishes or microtiter plates, such as had been previously done with mouse prostate fibroblasts (10). It soon became evident, however, that the effects of cell density on transformation (16, 79) were so major as to obscure the very meaning of the concept of transformation frequency. This was reinforced by early reconstruction experiments in which focus formation was measured after mixing of known numbers of nontransformed and transformed cells. Here it became evident that the numbers of foci in the dishes did not follow Poisson distributions, and that there was considerable migration of transformed cells to form satellite foci. Moreover, we found that the use of microtiter plates for transformation experiments, even with single cells, was not suitable because of variations in the background and indistinctness of the transformed foci that precluded their classification as Type I, II, or III, as originally defined and studied (16). It soon became clear that the enormous amount of time-consuming labor that went into the preparation and inspection of the dishes containing single cells, as well as the enormous number of dishes that would be required to obtain statistically significant results, precluded this approach to improved quantitation and scoring of this bioassay system. These complexities led to a theoretical and experimental examination of a probabilistic theory of transformed focus formation, which we believe will have major impact on the field. The manuscript, which has just been published (A. Fernandez, S. Mondal, and C. Heidelberger, Proc. Natl. Acad. Sci. USA 77: in press, 1980) is reproduced in its entirety in this report.

b. Quantitative Study of the Properties of Cells Derived from Various Types of Foci

In this study a rigorous investigation was made of various of the transformed phenotypes in a series of cell lines derived as defined below, in order to relate the "natural history" and evolution of these cultures to the phenomenon of tumor progression as defined by Foulds (49) and applied by Barrett and Ts'o (7) to the Syrian hamster embryo cell system. During the course of these laborious studies, a paper by Saxholm appeared (50), in which the same problem was approached from a different perspective with C3H/10T1/2 cells.

In a variety of cell culture systems an extremely tight correlation has been observed between the ability of a transformed cell to grow when cultured in semi-solid media (anchorage independence for growth) and its ability to grow as a tumor in vivo (48, 80-83). At the inception of this study, very little was known about the relationship between C3H/10T1/2 transformation and anchorage independent growth. The acute need for cell culture correlates with in vivo tumorigenicity prompted the initiation of this work.

The ability of cells to grow in semi-solid medium was assayed following a

modification of the basic procedures of Macpherson for soft agar culture (80). Briefly, cells were plated in 2 ml overlays of 0.3% agar or 0.25% agarose onto previously prepared 5 ml base layers of 0.5% agar or 0.4% agarose in 60 mm plastic dishes. Both overlays and base layers contained the equivalent of single-strength Basal Medium Eagle's (BME) supplemented with 10% fetal calf serum (FCS). To accomplish this, concentrated solutions of agar (Noble; Difco) or agarose (Type II; Sigma) were first prepared using double-deionized water. Aliquots of 45°C melted agar or agarose of the appropriate concentration were mixed with an equal volume of a 37°C mixture of 10X medium, serum and a sodium bicarbonate solution. This mixture was then pipetted in 5 ml aliquots into 60 mm dishes, allowed to harden and left in a biohazard hood (Baker) to dry overnight. Overlays were added the following day by first preparing the appropriate concentration of agar or agarose medium solution as just described, equilibrating this solution at 39.5°C in a water bath, and mixing equal volumes of this solution and a cell suspension prepared in complete medium. This mixture was then overlayed gently in 2 ml aliquots onto the base layers. Between $5x10^2$ and $1x10^5$ cells were plated per dish. The dishes were then incubated at 37°C in an atmosphere of 5% CO_2 in air for 3-4 weeks. To avoid drying, the dishes were fed once weekly by the gentle addition of 1-2 ml of complete medium. For scoring, cultures were examined microscopically and colonies of 16 or more cells counted. In an alternate scoring procedure, dishes were fixed in absolute methanol, stained with 5% Giemsa and colonies counted using a dissecting microscope.

The ability of cells to grow as tumors was assayed in syngeneic C3H mice. Mice were X-irradiated (450r) 24 hr prior to the injection of cells. Cells ($2x10^6$) were then inoculated subcutaneously (16) and the animals examined weekly for six months for the presence of tumors.

As shown in Table 1, the ability of transformed 58MCA Cl 16 cells to grow in various concentrations of agar or agarose overlays was compared. Base layers of 0.5% agar or 0.4% agarose were used in all studies. Preliminary studies (data not shown) had indicated that these base layer concentrations were the minimum required to prevent penetration of cells to the dish surface. Agarose was found to be superior for the culturing of 58MCA Cl 16 cells, permitting the formation of large colonies with a high plating efficiency. An agarose overlay concentration of 0.25% was found to be optimal. Higher overlay concentrations reduced colony sizes. Lower agarose concentrations were too dilute and colonies were observed to grow, migrate, fragment and fuse. Similarly, an agar overlay concentration of 0.3% was found to be optimal for soft agar culture.

Once the optimal conditions for soft agarose culture had been determined, C3H/10T1/2 Cl 8 and a number of transformed variants were assayed for anchorage independence of growth. These results are presented in Table 2. All eight tumorigenic cell lines tested grew in soft agarose culture. In general, all transformed cell lines fell into one of two classes with respect to the size of the colonies formed in soft agarose culture. Most of the cell lines grew to form only small colonies (25-200 cells) and are designated by a (+) in Table 2. Some cell lines grew to form very large colonies containing 1000 or more cells and are designated by a (++).

Of the five nontumorigenic cell lines tested here, three (including C3H/10T1/2 Cl 8) showed no, or very poor, capacity for growth in soft agarose. Two cell lines derived from Type I foci did grow in agarose, however. These cell lines grew to form very small

(25-50 cell) colonies.

The ability of a cell line to grow in soft agarose would appear to increase as a function of the number of passages it is maintained in culture. As is shown in Table 3, the plating efficiency and colony size of cells grown in agarose increase with passage number. The results with DMBA Cl III are particularly noteworthy. These cells were nontumorigenic when tested at passage 7, but were tumorigenic when tested at passage 15 (50). The acquisition of tumorigenicity in these cells appears to be accompanied by an increase in the ability to grow in soft agarose.

An experiment was then carried out to study the acquisition of various transformed phenotypes after treatment of C3H/10T1/2 cells as follows. Briefly, 2000 C3H/10T1/2 cells were seeded into 60 mm plastic petri dishes (Corning). After a period of 24 hr to permit cell attachment and the initiation of growth, dishes were medium-changed to medium containing 1 &g/ml of MCA and 0.2% acetone. Controls were treated with medium containing only 0.2% acetone. Treatment was terminated after 24 hr by medium-changing back to regular cell culture medium. The dishes were then medium-changed twice weekly until confluent, and once a week thereafter.

After the cultures had been confluent five weeks, the dishes were examined microscopically and the location of foci noted. Type II and/or Type III foci were observed in 34 of 55 treated dishes. No foci were found in 21 untreated dishes. Using cloning rings, four foci of every focus type were picked and transferred to mass culture. The four Type I cell lines were established from Type I foci found in dishes without Type II or III foci. Similarly, the four Type II cell lines were isolated from Type II foci found in dishes without Type III foci, and the four Type III cell lines were established from Type III foci. Finally, flat areas were picked from MCA-treated and acetone control dishes which did not contain foci and were respectively designated as Type 0 and Acet. The plating efficiencies on plastic and in agarose, the saturation density, and tumorigenicity, were determined for all cell lines at passage 3. The data are presented in Table 4. While all new cell lines grew well on plastic, only one Type II line displayed an ability to grow well in soft agarose. Three of four Type III derived cell lines displayed a minor amount of growth that was not deemed to be significant, but the fourth grew well. When colonies were seen, they were invariably (+) size. All of the new cell lines appeared to be nontumorigenic at passage 3.

These cells were then passaged further, and the morphology of the post-confluent cultures was examined at passages 5 and 7. As shown in Table 5, the acetone treated cultures retained the nontransformed morphology. However, the Type 0 cells (picked from flat areas of MCA-treated dishes) in some cases progressed to Types I and II. Type I cells progressed to Types II and III, Type II cells progressed to Type III, and Type III cells only gave rise to Type III progeny. This evolution of morphology was not reported by Saxholm (50).

The fact that none of these cells lines was tumorigenic at early passages, whereas 58 MCA Cl 16 was highly tumorigenic, suggests that oncogenic transformation occurs in several steps (7,49,50) or that the transformed cell lines are too strongly antigenic (12,19) to induce tumors in C3H mice that were immunosuppressed by 450 rad X-irradiation. We are now examining the feasibility of using intracranial injections or nude mice for tumorgenicity studies. The continuing evolution of these cell lines is being monitored, and the results will be published when we have obtained data at higher

passage numbers.

c. Morphology of Nontransformed and Transformed Cells in the Scanning Electron Microscope (SEM)

Initial SEM studies were hampered by malfunctions in equipment and rapid turnover of technicians. However, major progress has resulted from a collaboration with Dr. K.S. Narayan of the Pasadena Foundation for Medical Research, Pasadena, CA.

Cells grown on glass or plastic coverslips were processed for SEM studies according to procedures described previously (54). All procedures were carried out at room temperature unless otherwise mentioned. Cultures were rinsed with balanced salt solution (e.g. Dulbecco's saline G) and fixed in modified Karnovsky's fixative (2% glutaraldehyde, 1% freshly prepared formaldehyde, 1 mm $CaCl_2$ and 0.1 M cacodylate buffer, pH 7.4, osmolality of fixative approx. 360) at room temperature or at 37°C for 20-30 minutes. The cultures were then washed with 0.1 M cacodylate buffer containing 0.3 M sucrose and fixed with 2% OsO_4 in 0.1 M cacodylate buffer pH 7.4 for 1-2 hours. Following dehydration in ethanol, the samples were then critical-point dried from carbon dioxide with a Ladd critical-point dryer apparatus. The samples were sputter coated with gold-palladium in a Hummer V apparatus and scanned in an ETEC Autoscan at 20 kv with angles of 0-45°.

We have examined subconfluent cultures of C3H/10T1/2 cells taken at various times following treatment for 24 hours with 1.0 µg/ml of MCA. The surface morphology of untreated cells was flat and featureless (Figs. 1,2).

We have recently discovered that these carcinogen-treated cultures form a new transformation parameter, "mini-foci," which are seen also in cultures derived from Type II and Type III foci (see below). Mini-foci are groups of cells growing on top of each other in a criss-cross fashion and are detected under subconfluent culture conditions. They have been detected in MCA exposed cultures as early as 4 and 7 days following treatment (Figs. 3,4). Similarly grouped cells have also been found in 7 day cultures treated with MCA (0.1 µg/ml, 24 hours) alone or MCA (0.1 µg/ml, 24 hours) plus TPA (0.1 µg/ml, 1 hour, 4 days after MCA treatment) but apparently in much less frequency. Confirmation of these results and more precise quantitative determinations are planned for experiments with rigidly controlled culture conditions in which plating efficiencies can also be determined. Mini-foci have not been detected in control cultures.

SEM studies of carcinogen treated confluent cultures proved uninteresting and did not reveal differences in cell surface morphology that could be definitely identified as similar to or characteristic of transformed (Cl 16) cells. This was true even with cells in foci-like areas identifiable by light microscopy. Because of these rather disappointing results, we proposed to reexamine our assumptions and start with cells from foci of transformed cells detectable by light microscopy. We studied sublines of cells isolated from the three different types of foci - Type I, Type II and Type III. A "Type 0" subline, isolated from the "flat" areas between foci of cells in carcinogen exposed cultures, was also available. These, as well as controls (subline from acetone treated cells) were coded and processed for SEM examination. The cultures were seeded as usual (5000 cells per dish) and fixed for SEM at 4, 5 (subconfluent), 8 (confluent), and 14 days. The identity of each sample was checked only after completion of SEM examination.

SEM studies of these samples yielded encouraging results with subconfluent (4, 5 days) cultures. Cells with altered morphology and distinguishable from controls were easily seen in Type III and Type II cultures (Figs. 5,6). These cultures had cells that often occurred in groups and tended to overlap each other in a criss-cross fashion (Fig. 7). These "mini-foci" were taken to be significant in view of their similarity to the foci seen in the light microscope, and are considered to be diagnostic of transformed cells. Additionally, many cells in these early cultures showed a profusion of microvilli and blebs on their surfaces, and presented a morphology similar to Cl 16 or other oncogenic transformed cell populations. Type I cells (Fig. 8) were mostly non-overlapping and had fewer altered cells with microvilli and blebs, but their sparseness made scoring of these cultures as transformed difficult and equivocal. Type 0 cultures were similarly equivocal. Control, acetone treated cultures at similar subconfluency showed little or no detectable criss-cross cells, but contained cells with microvilli and ruffles. These cells at the relatively low (less than 400x) magnifications commonly used could not be distinguished as being different from those seen in the Type 0 and Type I cultures. Thus, distinctions between Type I, Type 0, and A (acetone) controls are equivocal and subjective. Experience suggests that quantitative analyses should be attempted in the future to make clear the distinctions, if any, among these cultures.

In other experiments, cells from Cl 16 (MCA-transformed, tumorigenic) and Cl 2 (DMBA-transformed, tumorigenic) cultures were examined after growth on glass or plastic surfaces under confluent and subconfluent conditions. The results of this study showed easily detectable cells with transformed morphologies in Cl 16 (Figs. 9,10) but not Cl 2 (Figs. 11,12) cultures. Subconfluent cultures were best for detection of the transformed cells. Cl 16 cells in comparison to control cells appeared less tightly adherent to the growing surface (more rounded). Cell surfaces often contained blebs and microvilli (Fig. 9). Microvillar distribution and size often appear to be heterogeneous. Cell criss-crossing was also noted. This overall morphology was maintained even in five-day-old cultures, a time at which control cultures are well-spread, actively growing and often nearly confluent. Confluent Cl 16 cultures were much less remarkable in appearance, had many fewer cells with large blebs or microvilli, and generally were difficult to distinguish from control 10T1/2 cells (Fig. 10). Nonetheless, closer examination showed subtle changes in cell-cell associations and cell shape.

Cl 2 (DMBA-transformed) cells were very different from Cl 16 (MCA-transformed) cells, and could not be characterized as tumorigenically transformed cells merely on the basis of their surface morphology. However, they were very different from control cultures in cell shape. These cells were typically epithelioid (Fig. 11), well-flattened, and formed close cell associations. Confluent, high density 14-day-old Cl 2 cultures showed some foci-like area of piled up cells with variable shapes and loose cell to cell associations (Fig. 12). Thus, although Cl 2 cells were not identifiable as transformed by virtue of their surface morphology, they were remarkably altered in terms of their cell to cell association pattern and cell shape.

SEM studies by Malick and Langenbach (55) with DMBA- and MCA-transformed C3H/10T1/2 cells have shown changes that are broadly similar to those described here. However, our studies have revealed some new findings not documented by previous authors. For example, the distinctive change of DMBA-transformed Cl 2 cells to an epithelioid shape in low density cultures seen in our studies has not been previously documented. Another significant new finding is the change in the tumorigenically transformed culture Cl 16 from cells with many surface alterations under subconfluent

conditions to predominantly smooth surfaced cells under confluent conditions.

Our studies on the SEM morphology of transformed C3H/10T1/2 cells obtained with sublines of Type I, II, and III foci is complemented by a recent study by Saxholm and Reith (56). These authors studied sublines obtained from foci of transformed cells after DMBA treatment and reported that "long" microvilli were preferentially found on Type II and Type III cells. They reported that Type III cells were homogeneous for the long microvilli, whereas Type II cells were heterogeneous. Their Type I subline did not show any cells with long microvilli. Our studies to date with MCA-transformed Type I, Type II, Type III, and Type 0 foci have given different results that are not confirmatory of Saxholm and Reith (56). However, we do not yet know whether such differences are attributable to the different chemical carcinogens used (DMBA vs. MCA) or other variables such as different culture conditions.

In summary, the experiments we have done so far allow us to conclude that transformed cells are recognizable by scanning electron microscopy at early times (within one week of carcinogen exposure) in terms of changes in cell surface morphology, and/or cell shape and/or cell to cell association (close, tight adhesion vs. loose contact; criss-cross, piled vs. parallel oriented, contact-inhibited). Secondly, we have seen that the expression of these changes is dependent upon culture conditions such as cell density. Thirdly, we have noted that treatment of C3H/10T1/2 cells with two different chemical carcinogens can result in dissimilar but transformed phenotypes.

d. Development of Monoclonal Antibodies to Oncofetal Antigens on Transformed Cells.

For the prototype experiments, two oncogenically transformed clones were chosen that had previously been shown to exhibit the oncofetal antigen (19): MCA Cl 15 and DMBA Cl 2. C3H mice were immunized 3 times at weekly intervals with 5×10^5 DMBA Cl 2 cells by intraperitoneal injections and were bled 3 days after the last immunization. The binding capacity of the antibodies to the transformed cells was determined by the binding of protein A. This protein, which is a single polypeptide isolated from the cell wall of Staphylococcus aureus, has an extraordinary affinity of binding to the Fc region of the immunoglobulin IgG (77). Thus, the assay measures the amount of protein A (labeled with ^{125}I) that binds to the Fc portion of the antibody, which in turn binds to the surface antigen of the intact cell through the Fab portion of the antibody. The binding assay was carried out by the method of Binz and Wigzell (85). The transformed cells (1×10^5) were plated in Falcon 96 well microtiter plates and allowed to attach for 12 hr. They were then washed 3 times with Basal Medium Eagle's (BME) with 1% bovine serum albumin. The cells were then treated for 30 min at 4°C with the mouse antiserum or various dilutions thereof in BME. The cells were again washed 3 times with BME and incubated for 1 hr at room temperature with 10^5 cpm of ^{125}I-protein A (Pharmacia) radioiodinated with chloramine T (86) in phosphate buffered saline (PBS), and then washed 3 times with PBS. The wells of the microtiter plate were sawed out, placed in liquid scintillation vials, and the bound ^{125}I was counted. The results are shown in Table 6. The antiserum obtained from DMBA Cl 2 immunized mice bound to those cells and to MCA Cl 15 cells to approximately the same extent and to a significantly higher extent than to the non-immune serum control at dilutions of 1:5. This similarity in extents of binding suggests that the antibody bound to a common cell-surface antigen on both transformed cells.

The experiment shown in Table 7 demonstrates that there was no significant

binding of nontransformed C3H/10T1/2 Cl 8 cells to antisera prepared against those cells or to the antiserum prepared against the transformed DMBA Cl 2 cells. Moreover, the transformed DMBA Cl 2 cells reacted with the antiserum directed against the same cells, but not with the antiserum to the nontransformed cells. These two experiments demonstrated that the cells and assays used were sufficiently selective and sensitive to allow us to proceed to prepare monoclonal antibodies from hybridomas as first described by Kohler and Milstein (71).

C3H mice were immunized 3 times once weekly with $5x10^6$ DMBA Cl 2 transformed cells. Three days after the last immunization, spleen cells were collected and fused with a mouse myeloma cell line, P3 x 63/Ag8, that lacks thymidine kinase (obtained from Dr. J. Frelinger) according to the method of Galfre et al. (87), using polyethylene glycol. After completion of the cell fusion and selection in HAT medium (hypoxanthine, aminopterin, and thymidine) for two weeks, the cells were then cultured in regular medium. After the fourth week in culture, the supernatants of the hybridomas were added to target DMBA Cl 2 cells plated in microtiter wells as described previously and assayed by ^{125}I protein A binding as also described previously. The wells were either counted for ^{125}I binding, or the pattern of bound ^{125}I was determined by autoradiography at -70°C. In a typical experiment (Fig. 13), 4 out of 120 hybridoma wells contained antibodies against the transformed cells. These were designated as C1, B4, B5, B6 (Panel 1, Fig. 13). Further cloning of 300 cells derived from the B5 well that originally contained $1x10^5$ cells into another microtiter plate revealed that the supernatants from 6 wells (A5, A6, A7, B3, E7, C11) were reactive to the transformed cells after one week in culture. However, when the cultures were continued for an additional week, only one well (C11) remained positive, and its supernatant reacted with both transformed clones but not to the nontransformed clone. This suggests that immunoglobulin synthesis was unstable in some hybridomas, as reported by many other laboratories (cf. 88). In another cell fusion experiment (Fig. 14), 3 wells (X4Y3, X6Y3, X6Y2) out of 120 contained hybrid cells that secreted antibodies that bound the two transformed, but not the nontransformed cell lines. Other hybridomas that secrete antibodies (possibly TSTA or others) can also be visualized. Experiments are now in progress to prepare pure clones of the hybridomas that secrete antibodies of the desired properties.

e. Specific Killing of Transformed Cells by Activated Peritoneal Macrophages.

The first approach to this problem involved setting up an assay for the killing effect of activated peritoneal macrophages. Since much of our work involves determination of the reproductive capacity of cells by measuring their plating efficiency, and since this assay has not been applied to macrophage killing, we decided to use this method and compare it with the ^{125}IUdR release assay (77,78).

We activated the peritoneal macrophages of C3H mice by injecting IP 1 ml of BCG containing 2×10^7 live organisms. Nine days later, the mice were injected IP with 2 ml of thioglycollate 5 days before harvesting the macrophages. After harvesting, the cells were counted and plated in 100 mm dishes ($2x10^6$/dish) with 10 ml of Basal Medium Eagle (BME) + 10% fetal calf serum (FCS). After one hr incubation, the medium was aspirated out; the adherent cells are practically all (90-95%) macrophages. We then plated 5000 of our test cells with 10 ml of BME + 10% FCS and incubated for 48 hr. At the end of this time we scraped all the cells from individual dishes and replated, without counting, into 10 100 mm dishes with 10 ml of medium in each. The dishes were incubated for 10 days and fixed with methanol, stained with Giemsa and

scored for the development of colonies. The results are shown in Table 8, which demonstrates that the activated macrophages recognize the malignant DMBA C1 2 and MCA C1 16 cells and kill them, as shown by the decrease in number of colonies (42 and 45%). In contrast, the nontransformed C3H/10T1/2 cells were not killed by the macrophages and the number of colonies was actually increased as compared to the dishes without macrophages (195%). This is due to a feeder effect.

An exactly comparable experiment was then set up with the nontransformed C3H/10T1/2 C1 8 cells, and with acetone-treated and Types 0, I, II, and III-derived cell lines at passage 9 (these cell lines were defined in Progress Report, part e). None of these cell lines at this passage number grew significantly in soft agarose. As shown in Table 9, the activated macrophages again enhanced the plating efficiency of the C3H/10T1/2 C1 8 cells, but produced no significant effect on the reproductive capacity of the others except for a marginal killing effect (82%) on the Type III cell line.

Dr. Michael Fisher of the Frederick Cancer Center has performed on our cells a newly developed microassay (89), which is carried out in 96-well microtiter plates (Falcon). Log phase target cells are pre-labeled for 24 hr with 0.2 μCi/ml of ^{125}IUdR (New England Nuclear, 200 mCi/mMol), washed 3 times, and trypsinized and counted. Mouse peritoneal macrophages were activated in culture with macrophage activating factor (MAF) (78) for 24 hr. Then 10^5 viable (trypan blue) macrophages (MP) are added to each microtiter well. After 45 min the macrophages were washed 3 times to remove nonadherent cells, and 5×10^3 target cells were added to each well so that the ratio of macrophages to target cells added was 20:1. After one day, the cells were washed to remove nonadherent cells, and incubation was carried on for 3 additional days when the cells were washed three times and incubated for 1 hr with 0.1 N NaOH, and the radioactivity of the NaOH lysate was determined in a gamma counter. In all experiments, controls were run in which the target cells were incubated with nonactivated peritoneal macrophages. The target cells, the same as those described in Table 9, were sent to Dr. Fisher at the 9th passage, and were tested by him at the 14th passage. The results are shown in Table 10. The percent cytotoxicity is calculated according to the equation

$$\% \text{ Cytotoxicity} = 100 \times \frac{\text{cpm } ^{125}\text{I in target cells with nonactivated MP} - \text{cpm } ^{125}\text{I in target cells with activated MP}}{\text{cpm } ^{125}\text{I in target cells with nonactivated MP}}$$

In these experiments there was no cytolysis produced by the nonactivated macrophages of any of the cell lines, or by the activated macrophages on the nontransformed C3H/10T1/2 C1 8, acetone-treated, or Type I cells. However, there was significant lysis of the Types II and III cells, as well as of the oncogenically transformed MCA C1 16 cells. It is not known whether the differences in results between these experiments (Table 10) and the ones done by plating efficiency (Table 9) are due to the difference in the assay, the difference in the method of activation of the macrophages, or the difference in the passage number of the Types II and III cells. It is clear, however, that the micro ^{125}IUdR assay is quicker, easier, requires less cells and media, and is more accurate than the plating efficiency assay. Hence, all future experiments will be done in our laboratory by the micro radioactivity assay.

Table 1

Growth of 58MCA Cl 16 Cells in Agar or Agarose:

Effect of Overlay Concentration

Suspending Material	Growth Property	Overlay Concentration 0.2%	0.25%	0.3%	0.35%
Agar	Plating Efficiency[1] (%)	ND[2]	12%	12%	5%
	Range of Colony Sizes	ND[2]	0.03-0.3mm	0.03-0.3mm	0.03-0.1mm
Agarose	Plating Efficiency[1] (%)	27%	27%	19	ND[2]
	Range of Colony Sizes	0.3-1.0mm	0.3-1.0mm	0.03mm	ND[2]

Cells were plated in agar or agarose suspension culture as described. Agar base layers were always 0.5%, agarose base layers were 0.4%. For each condition, 6 dishes containing 500 cells were seeded per experiment. Each experiment was performed twice.

1. Plating Efficiency (PE) is calculated as the % of cells seeded which grow to form colonies.

2. ND - Not Done

Table 2

Plating Characteristics and Tumorigenicity of

C3H/10T1/2 Cl 8 and its Transformed Variants

Cell Line	Passage Number	PE on Plastic (%)	PE in Agarose (%)	Colony Size[1]	Tumorigenicity[2]
C3H/10T1/2 Cl 8	5-15	20-30%	.001	-	-
58MCA Cl 13	23	30	4.5	++	+
58MCA Cl 14	27	28	2.5	+	+
58MCA Cl 15	8	20	2.4	+	+
58MCA Cl 16	39	31	26.5	++	+
DMBA Cl D	45	38	23.1	+	+
DMBA Cl III	7	29	0.27	+	-
DMBA Cl H	32	32	38.7	++	+
DMBA Cl II	15	37	0.06	+	-
DMBA Cl I	7	36	13	+	-
DMBA Cl K	45	18	25	+	-
UVTX-1	2	25	4.9	+	+
UVTX-2	2	17	4.7	+	+

All experiments were performed in duplicate.

1. (+): indicates colonies generally smaller than 0.2 mm.
   (++): indicates colonies generally larger than 0.2 mm.

2. Tumorigenicity data provided by the investigators who isolated the cell lines.

Table 3

Effect of Passage Number on the Ability of a Cell Line to Grow in Soft Agarose

Cell Line	Passage Number	PE on Plastic (%)	PE in Agarose (%)	Colony[1] Size
C3H/10T1/2 Cl 8	5	28	.001	-
	20	25	.001	-
58MCA Cl 16	8	25	4.5	+
	25	28	16	++
	39	31	27	++
DMBA Cl III	7	29	0.27	+
	15	33	1.7	+
58MCA Cl 13	7	27	1.2	+
	23	30	4.5	++

All experiments were performed in duplicate (5-6 dishes per line per experiment).

[1] Sizes are as described in Table 2.

Table 4

Characterization of Clones Isolated from MCA Experiment

Orig. Type	Clone ω	Passage ω	Type	Plastic PE (%)	Agarose PE (%)	Sat. Density/ 60mm Dish	Tumors
Acet	1	3	N	29	.001	9.1×10^5	0/4
Acet	2	3	N	32	.001	9.6×10^5	0/4
Acet	3	3	N	31	.001	1.0×10^6	0/4
Acet	4	3	N	27	.001	9.0×10^5	0/4
0	1	3	1	30	.001	1.8×10^6	0/4
0	2	3	N	17	.001	9.3×10^5	0/4
0	3	3	N	22	.001	9.2×10^5	0/4
0	4	3	N	24	.001	1.2×10^6	0/4
I	1	3	1	24	0.08	1.4×10^6	0/4
I	2	3	2	22	.01	3.1×10^6	0/4
I	3	3	1	25	.01	9.6×10^5	0/4
I	4	3	N	25	.01	1.1×10^6	0/4
II	1	3	3	24	0.05	1.7×10^6	0/4
II	2	3	3	24	0.3	4.3×10^6	0/4
II	3	3	2	23	0.08	1.2×10^6	0/4
II	4	3	2	22	0.08	1.2×10^6	0/4
III	1	3	3	21	0.11	1.8×10^6	0/4
III	2	3	3	29	27	3.3×10^6	0/4
III	3	3	3	26	0.20	3.5×10^6	0/4
III	4	3	3	17	0.28	2.0×10^6	0/4
58 MCA	16	32	3	34	37	4.3×10^6	4/4

Table 5

Evolution of Post-Confluent Morphology with Passage

Orig. Type	Clone ω	Morphology at Passage No. 3	5	7
Acet	1	N	N	N
Acet	2	N	N	N
Acet	3	N	N	N
Acet	4	N	N	N
0	1	1	1	2
0	2	N	N	1-2
0	3	N	1	1-2
0	4	N	N	1
I	1	1-2	2	2
I	2	2	3	3
I	3	1	2	2
I	4	N	1	1
II	1	3	3	3
II	2	3	3	3
II	3	2	2	3
II	4	2	3	3
III	1	3	3	3
III	2	3	3	3
III	3	3	3	3
III	4	3	3	3

Table 6

Antigenic Cross-Reactivity of Oncogenically Transformed C3H/10T1/2 Cells as Determined by the ^{125}I Protein A Assay.

Transformed Cell Line	Antiserum	Cpm Bound Protein A
DMBA Cl 2	None	276 ± 10
"	Non-immune (1:1)	1,394 ± 101
"	Immune (1:1)	34,920 ± 263
"	Immune (1:5)	7,892 ± 113
"	Immune (1:25)	2,189 ± 392
"	Immune (1:125)	722 ± 45
MCA Cl 15	None	360 ± 18
"	Non-immune	2,063 ± 99
"	Immune (1:1)	32,158 ± 149
"	Immune (1:5)	8,997 ± 1,651
"	Immune (1:25)	2,415 ± 166
"	Immune (1:125)	862 ± 97

Table 7

Binding of Nontransformed and Oncogenically Transformed Cells to Various Antisera

Target Cell Line	Antiserum at 1110	Cpm Bound Protein A
C3H/10T1/2 Cl 8	Non-immune	3,005 ± 78
" "	Anti-C3H/10T1/2 Cl 8	3,166 ± 483
" "	Anti-DMBA Cl 2	3,609 ± 86
DMBA Cl 2	Non-immune	3,278 ± 45
"	Anti-C3H/10T1/2 Cl 8	3,278 ± 45
"	Anti-DMBA Cl 2	11,886 ± 1,652

Table 8

Effect of Macrophages on Plating Efficiencies of Cells

	Total colonies	Ave. Colonies/ dish	PE (%)	Ratio
C3H/10T1/2 Cells	1425	119	14	
C3H/10T1/2 Cells + Macrophages	2787	232	28	1.95
DMBA Cl 2 Cells	4678	390	47	
DMBA Cl 2 Cells + Macrophages	1958	163	20	0.42
MCA Cl 16 Cells	4628	386	46	
MCA Cl 16 Cells + Macrophages	2061	172	21	0.45

Table 9

Effects of Macrophages on Cells Following MCA Treatment
(None of the cell lines grew in soft agarose)

Cell	Passage No.	Total Colonies	Colonies/ Dish	Ratio
C3H/10T1/2 Cl 8	8	6201	310	
" + Macrophages	8	9316	466	1.5
Acetone	9	7216	361	
" + Macrophages	9	7628	381	1.1
Type 0	9	3876	388	
" + Macrophages	9	3696	370	0.95
Type 1	9	7621	381	
" + Macrophages	9	8334	417	1.09
Type 2	9	6924	346	
" + Macrophages	9	6279	314	0.91
Type 3	9	5310	266	
" + Macrophages	9	4338	217	0.82

Table 10

Percent Cytotoxicity in ^{125}IUdR Release Microassay
(Conditions were as described in the Text)

Cell Line	Passage No.	Expt. 1	Expt. 2
C3H/10T1/2	12	4	5
MCA Cl 16	35	20	22
Acet.	14	NT	0
Type 1 Cl 2	14	NT	2
Type 2 Cl 3	14	30	34
Type 3 Cl 1	14	45	28

NT = Not Tested

Legends for Figures

Figures 1,2.

Scanning electron micrographs for nontransformed C3H/10T1/2 cells in subconfluent (Fig. 1) and confluent (Fig. 2) cultures. Note cells are very flat, thinly spread and often in close juxtaposition. Cell surfaces appear generally smooth. Both figures at same magnification. Bars represent 10 μ.

Figures 3,4.

Scanning electron micrographs illustrating mini-foci (groups of cells in criss-cross patterns) at early times following carcinogen (MCA) treatment of C3H/10T1/2 cells. Figure 3 is from a subconfluent culture, 4 days after MCA (1.0 μg/ml, 24 hour) treatment. Figure 5 is from an older culture, treated with MCA (0.1 μg/ml, 24 hours) and TPA (0.1 μg/ml), 1 hour at 3 days after MCA treatment), and fixed 7 days after MCA treatment. Bars represent 10 μ.

Figures 5,6.

Scanning electron micrographs illustrating transformed surface morphology commonly seen cells derived from Type 3 and Type 2 foci. Both figures are from 5 day-old cultures and are shown at the same magnification. Bars represent 10 μ.

Figure 7.

Scanning electron micrograph illustrating mini-foci in a 7 day old Type 3 culture, at near confluency. Bar represents 10 μ.

Figure 8.

Scanning electron micrographs illustrating long and short microvilli in cells of a 5 day old Type 1 culture. Bar represent 10 μ.

Figures 9,10.

Scanning electron micrographs of oncogenically MCA-transformed (Cl 16) cells showing different morphologies under subconfluent (Figure 9) and confluent (Figure 10) conditions. These two photographs were from areas found on the same coverslip. Culture, 7 days old. Photographs shown at same magnification. Bars represent 10 μ.

Figures 11,12.

Scanning electron micrographs at the same magnification of DMBA transformed (Cl 2) cells illustrating different morphologies under subconfluent (5 day old culture, Figure 11) and confluent conditions (14 day old culture,

Figure 12). Bars represent 10 μ.

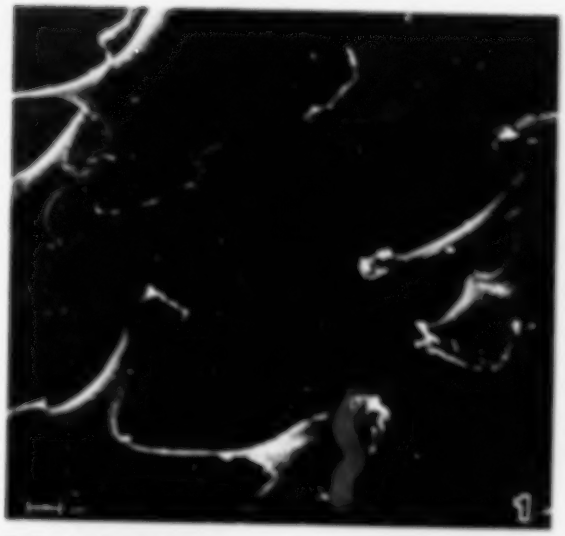
Figure 13.

Detection of hybrid cells that secrete antibodies against DMBA C12 antigens. The hybrid cells were obtained as described above. The wells containing antibodies were detected autoradiographically by the amount of ^{125}I-protein A bound. Then the wells were sawed out and the radioactivity counted, as shown in the figure. The positive wells were designated (Panel I) as C1, B4, B5, and B6. The cells from well B5 were further cloned, and (Panel II) wells A5, A6, A7, B3, C11, and E7 were positive at one week. After an additional week, only well C11 (Panel III) remained positive.

Figure 14.

Autoradiographic demonstration of hybrid cells secreting antibodies against common transformation antigens.

Hybridomas were obtained, as described for Fig. 13 from mice immunized with DMBA C1 2. Plate I plus the upper part of Plate II (see lines) contained DMBA C1 2 (D2) target cells. The lower part of plate II and the upper part of plate III contained MCA C1 15 (M15) target cells. The lower part of plate III and plate IV contained C3H/10T/2 (10T1/2) target cells. The hybridoma supernatants were distributed identically to the three target cells i.e. the supernatant from hybridoma originally in X1Y1 was added to well X1Y1 containing the different target cells in each plate. Those antibodies that reacted with both transformed clones and not with the nontransformed clone, were found in wells X4Y3, X6Y3, and X6Y2.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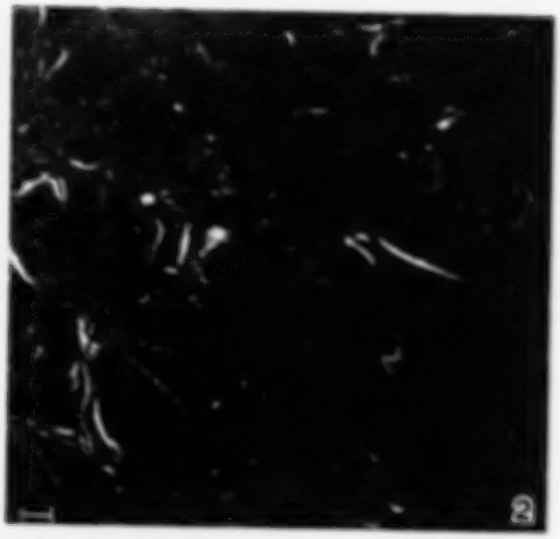
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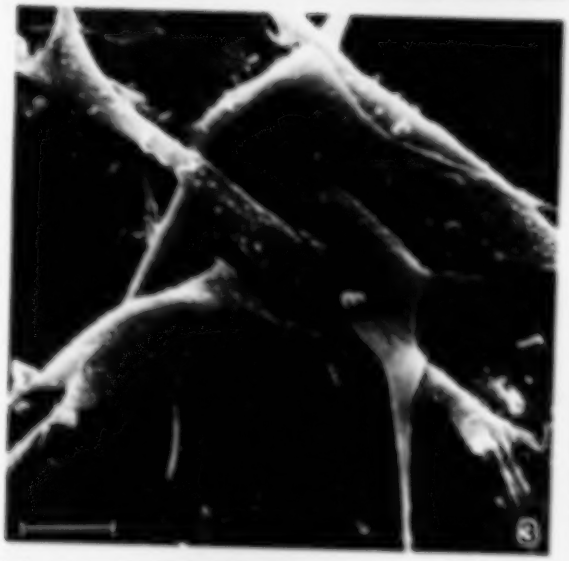
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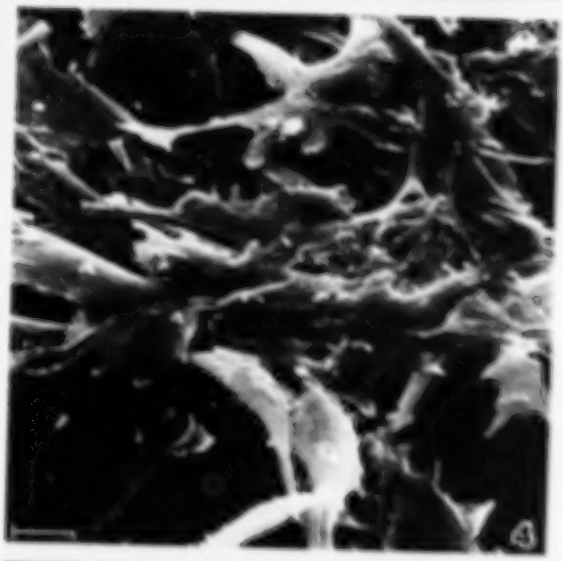
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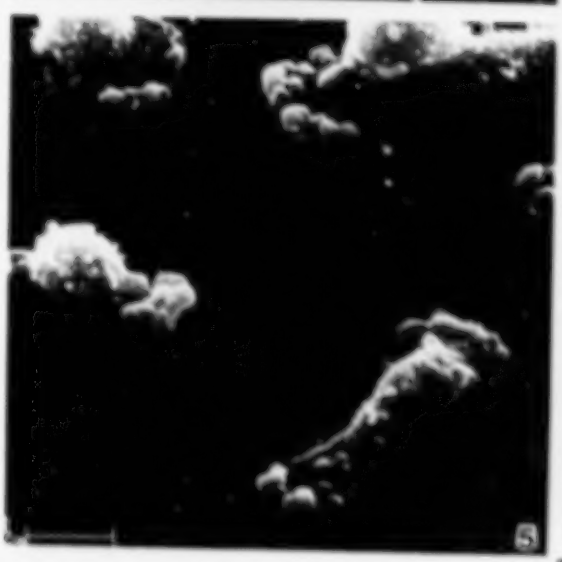
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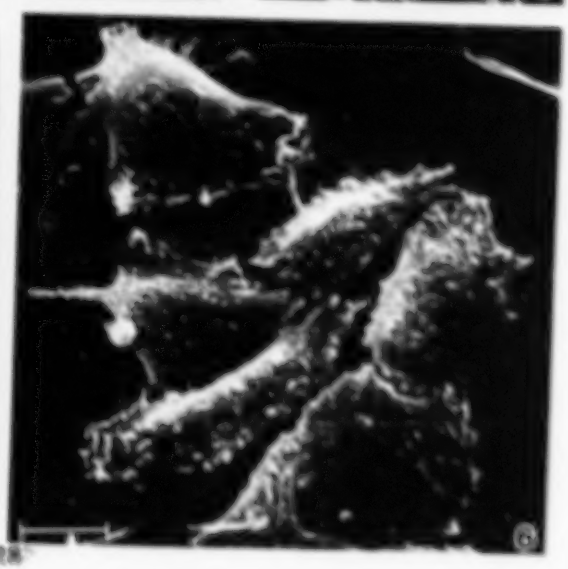
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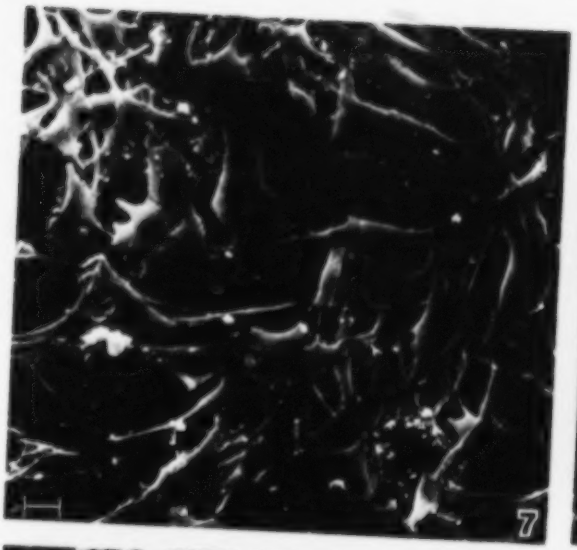
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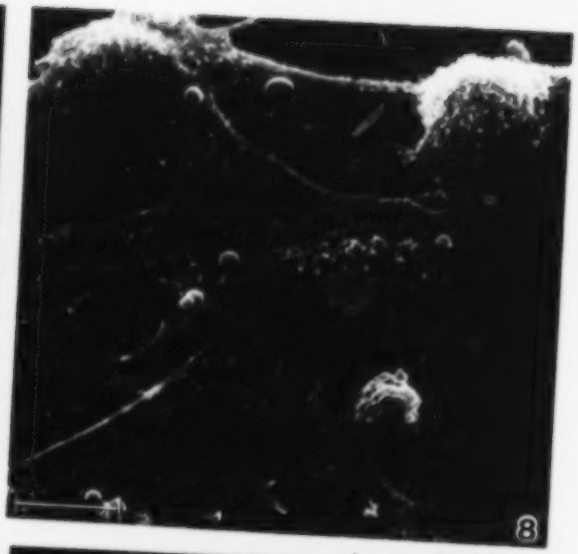
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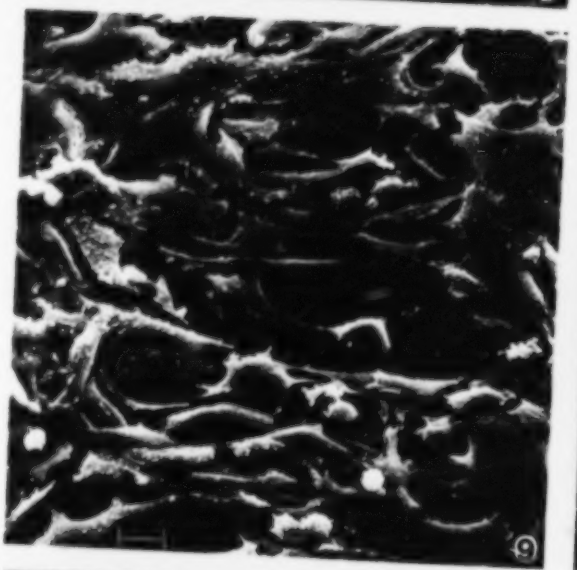
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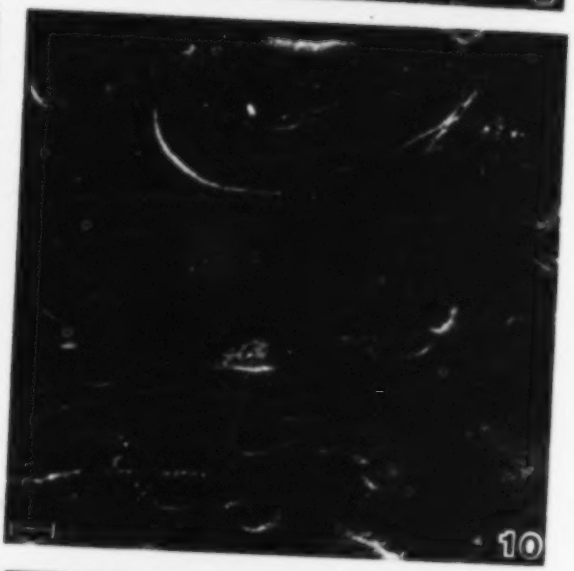
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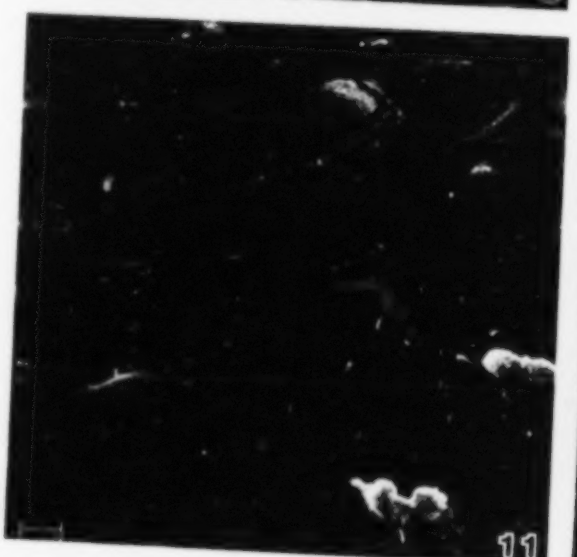
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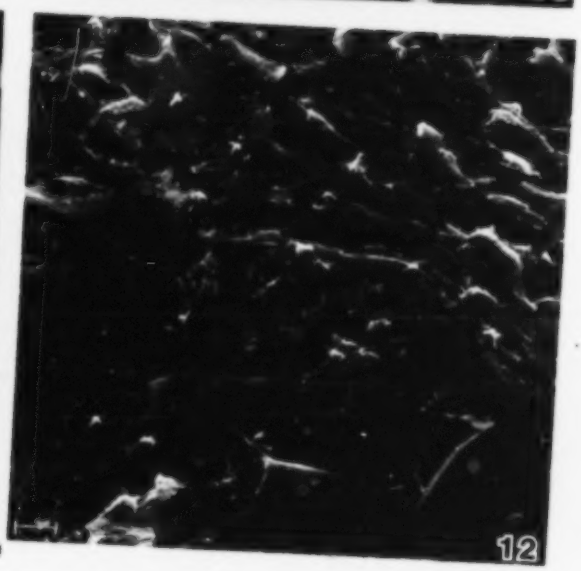
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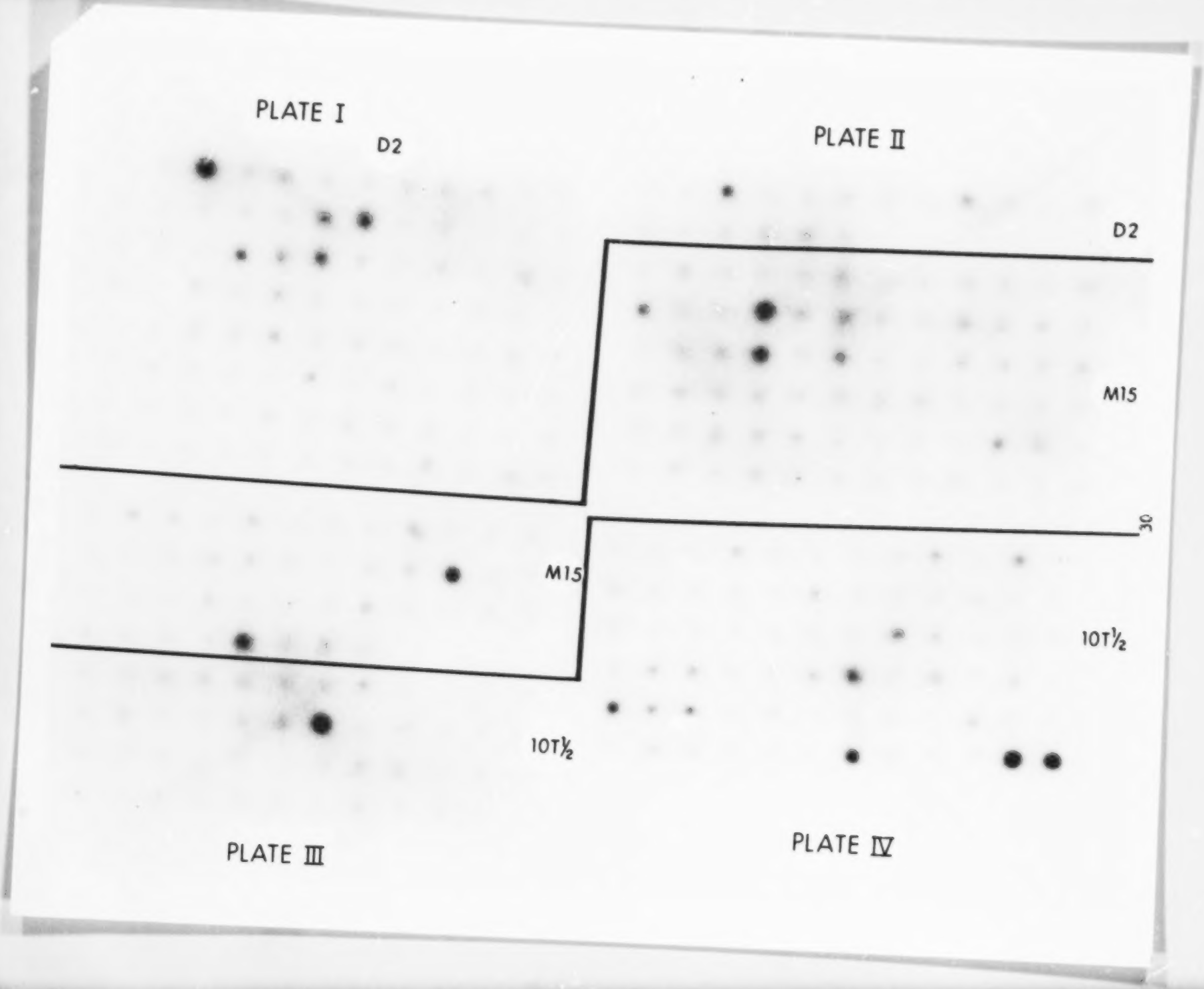
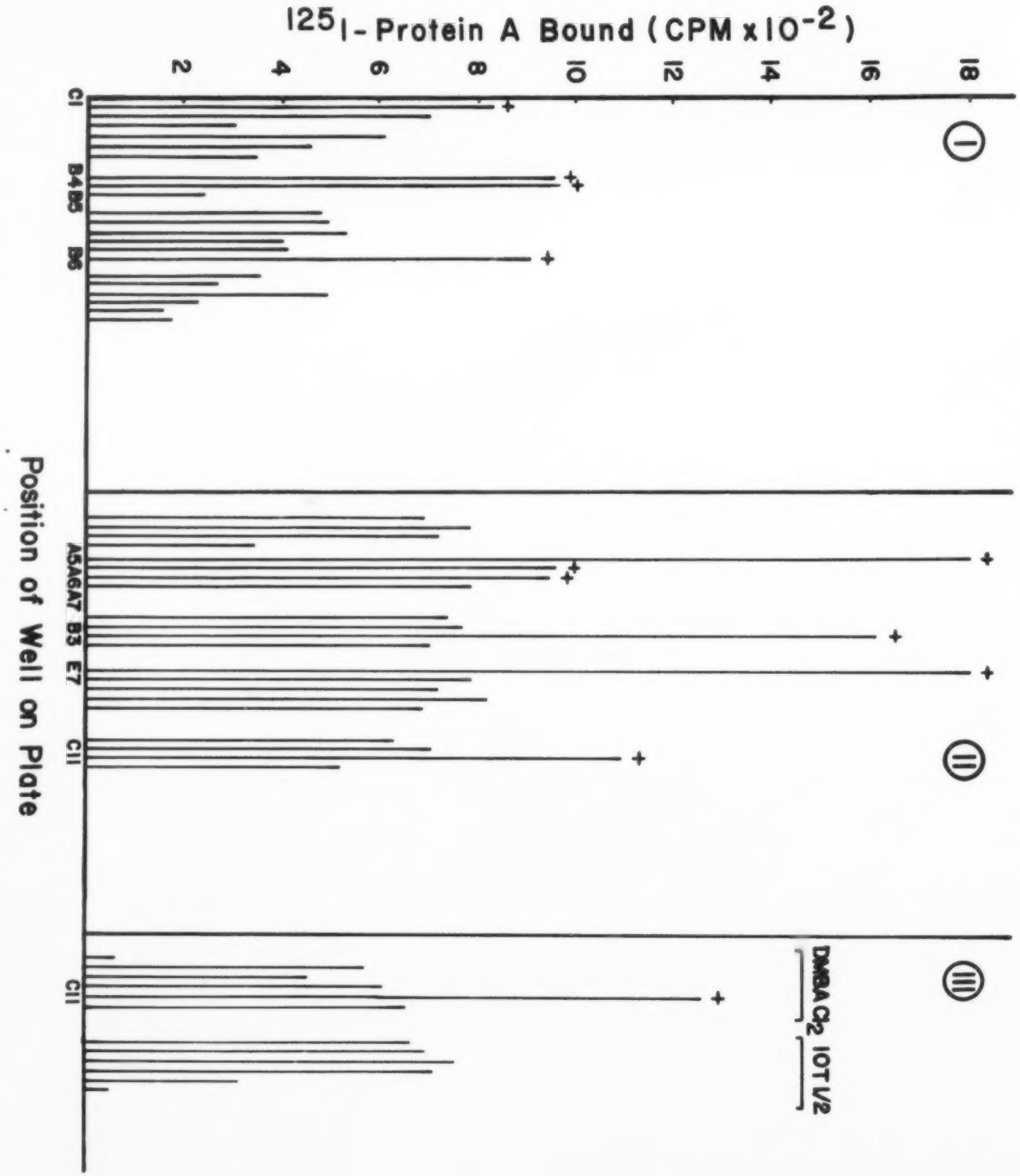


PLATE I
D2
PLATE II
D2
M15
30
M15
10T½
10T½
PLATE III
PLATE IV


^{125}I-Protein A Bound (CPM x 10^{-2})
2
4
6
8
10
12
14
16
18
C1
B4 B5
B6
A5 A6 A7
B3
E7
C11
C11
I
II
III
DMBA Cl_2 10T 1/2
Position of Well on Plate

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